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MICRODISSECTION AND MEASUREMENT OF POLYTENE CHROMOSOMES USING THE ATOMIC FORCE MICROSCOPE

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Abstract

A method to isolate specific regions of the *Drosophila* polytene chromosome using an atomic force microscope (AFM) was explored. The AFM was used for the microdissection of the locus of interest with much greater precision than standard microdissection techniques. The amplification of DNA isolated in this fashion by the polymerase chain reaction (PCR) is discussed. A study of the effect of hydration level on gross chromosome structure was carried out. It was shown that chromosome swelling is dependent upon humidity or the buffered medium. The significance of this swelling with respect to studies of chromosome structure under physiological conditions is considered.

Key Words: Atomic Force Microscope, polytene chromosome, microdissection, humidity.

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Introduction

Since the invention of the atomic force microscope (AFM, [1]) and its eventual use in structural biology (for reviews cf. [5, 6, 8]) an important area of research has focused on the use of the AFM as a micromanipulation tool. Initial efforts at microdissection of plasmid DNA in air [4, 19] and propanol [3] have proved successful at making precise (20 nm wide) cuts. Henderson has shown that the AFM can be used not only to dissect but manipulate the excised fragment from plasmid DNA [4].

Larger biological structures have also been successfully dissected with the AFM, including gap junctions [9, 11] and living cells [6, 7, 13, 14]. An important and well characterized biological sample that lends itself to the development of the AFM as a microdissection tool is the polytene chromosome. These unusually large chromosomes have been routinely dissected using glass needles, and PCR amplified using degenerate primers [20]. Standard glass needles are typically 500 nm in width, thus limiting the dissection path to similar dimensions. However, AFM tips may be as sharp as 20 nm at the apex. The greater precision afforded by the AFM could provide a significant refinement in microdissection methods. In this paper, we examine the utility of the AFM as a potential microdissection tool for isolation and molecular characterization of chromosomal DNA fragments.

In an effort to facilitate dissection of chromosomes, we explored the relationship between susceptibility to scission and hydration state. During the course of these studies, we found that chromosomes swell as a function of humidity, as reported earlier by other investigators [2]. Interestingly, preliminary studies of the effect of the chemical environment suggest that chromosome swelling is very dependent upon variables such as pH and ionic strength. Further studies in this area may broaden our understanding of the fundamental question of higher order chromatin packing by taking advantage of the range of forces that characterize probe sample interactions [17].

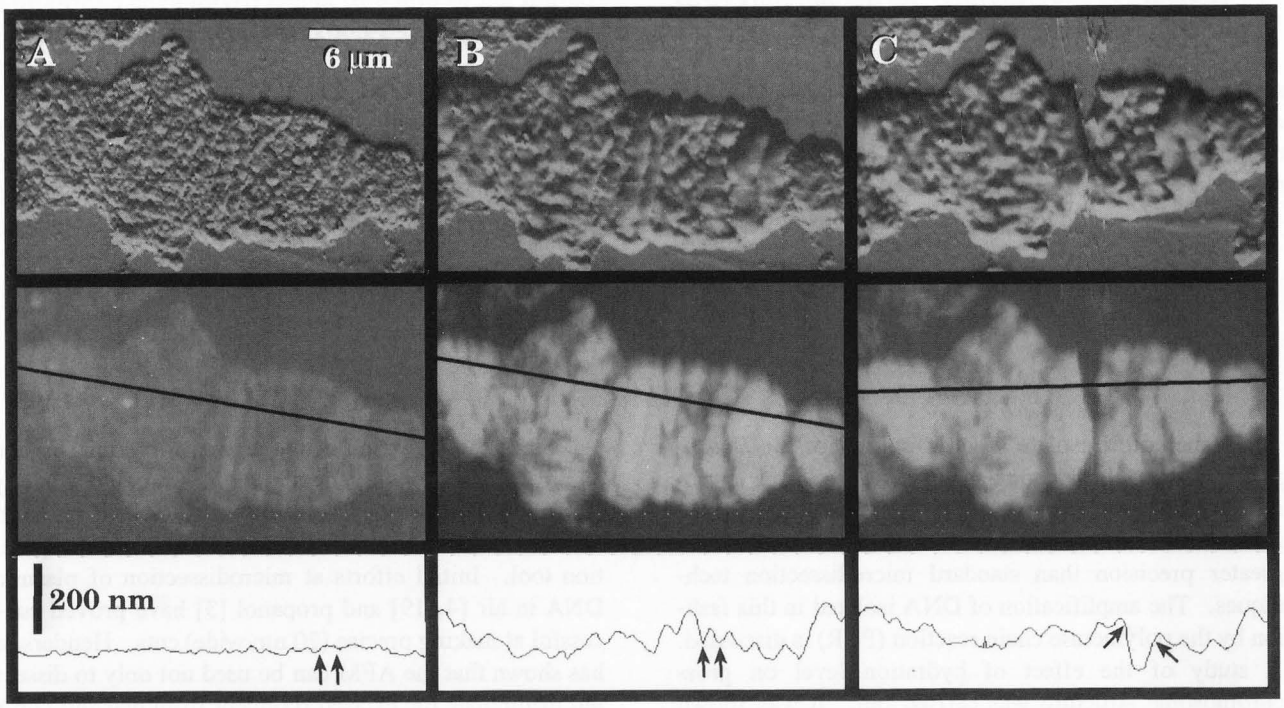

Figure 1. Height (middle row), error signal mode (ESM, top row) and representative height section (bottom row) images of *Drosophila melanogaster* polytene chromosomes prior to and immediately following microdissection. Column **A**, images in dry air at RH < 20%. Column **B**, same chromosome imaged under phosphate buffered saline (pH 7.5) showing band details, allowing selected loci identification. Post-microdissection images (column **C**) enable assessment of the cutting procedure. The appearance of chromosomal debris near the cut, especially evident in the ESM, shows the removal of chromosomal material. Also, the loss in scan resolution after cutting may be attributed to the accumulation of excised DNA and nucleoproteins on the tip.

Materials and Methods

Drosophila melanogaster salivary gland chromosomal squashes were prepared from a locally inbred stock using the procedure of Pardue and Gall [12]. The 1E region near the end of the X chromosome was selected for dissection due to its ease of identification. The NanoScope III Bioscope (Digital Instruments Inc., Santa Barbara, CA) was used for all of the sample imaging and microdissections. The Bioscope is a combined optical/atomic force microscope designed for imaging biological samples under physiologically relevant conditions. The AFM portion has a full compliment of dry and wet cell, contact and non-contact imaging. An Axiovert 135 (Carl Zeiss, Thornwood, NY) with epifluorescence optics and ultra-violet (UV) light source provided the optical component. This is further integrated with a scanning laser confocal microscope (Noran Instruments, Middleton, WI). Thus, the Bioscope combines the high resolution, three dimensional imaging/manipulation capabilities of the AFM with the well established imaging capability of a confocal optical microscope.

Silicon nitride cantilevers (200 μm long, with a nominal spring constant of 0.06 N/m), mounted in a fluid cell, were used to obtain AFM images. The 1E region of a chromosome, that was suitable for dissection, was located optically. This region was then imaged using the AFM in contact mode in dry air (relative humidity < 20%) and with an applied vertical force typically less than 10 nN measured from tip-sample breakaway. Images were recorded in both height and error signal modes (ESM) [16].

For the purposes of chromosome dissection, the tip was withdrawn and several drops of phosphate buffered saline (PBS; pH 7.3) were added to the sample surface. The sample was allowed to hydrate for 30 minutes. At engagement, the user adjustable force was minimized (less than 3 nN from zero cantilever deflection as no breakaway force was observed in PBS buffer). The scan size and scan angle were adjusted until only the chromosome of interest was being scanned and was oriented with bands parallel to the fast scan axis (adjustment

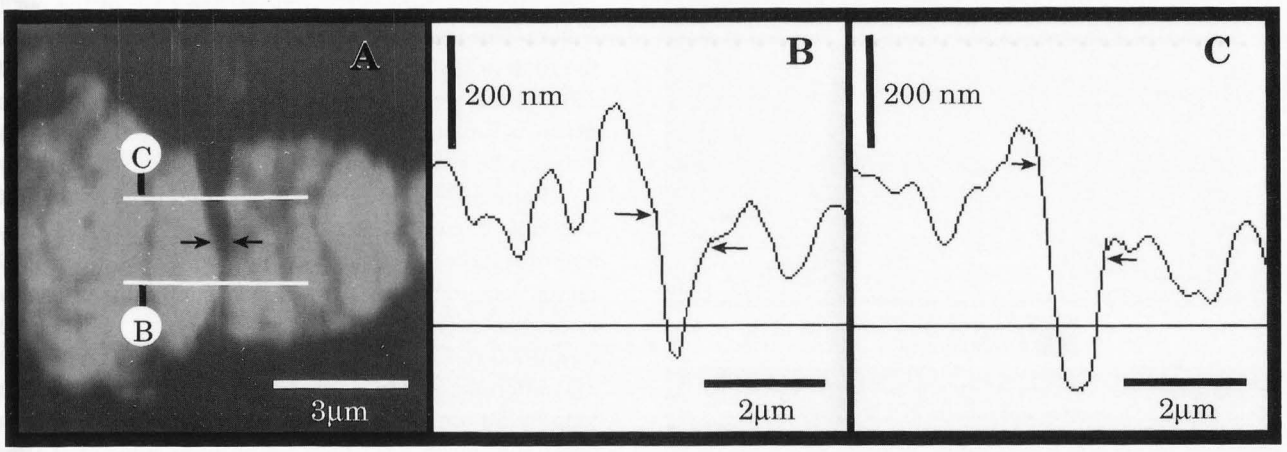

Figure 2. Height mode image (**2A**) of the 1E region of a *Drosophila* chromosome with parallel white lines used for cross-sectional analysis. Line B is a section through the end of the cut where the tip is not in contact with the glass substrate (**2B**). Line C is a cross-section through the middle of the cut where the tip makes contact with the glass substrate (**2C**). The wedge shape of the cut is due to change of the scan orientation while cutting was underway. The shape of the tip is evident in the walls of the cut, making an angle of 30° as compared to a half angle of about 26° for commercially made oxide-etched pyramidal silicon-nitride probes. These cross-sections suggest highly precise dissection, from tens of nanometers to micrometers, depending on the sample height and scan orientation is possible.

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observable in Fig. 1C). An image was captured (scan rate 80 μm/sec) to allow band identification, and the slow scan axis was disabled when the tip crossed the band selected for microdissection. The gains were reduced, the scan speed was increased (320 μm/sec), and the force was maximized (approx. 100 nN). After several minutes, the settings were returned to their original values, but they were changed in the opposite order. Again, an image was captured to show the chromosome following band dissection.

The microdissection process is routine and has been performed on a dozen samples. The ease of chromosomal dissection varied between samples (presumably based on squash preparation) but was mitigated by hydration in PBS buffer.

Results and Discussion

Microdissection of the sample was accomplished by orienting the bands parallel to the fast scan axis (Figure 1). Figure 1A is the sample imaged in dry air; the top row represents detailed error signal mode (ESM) [16] images, the middle row shows height images and the bottom row shows typical cross-sections through the dissected regions. After selection of a banded region for cutting in buffer solution (Figure 1B), the slow scan axis was disabled and the force increased to enhance microdissection of the specimen. After several minutes, the force was reduced and the slow scan axis enabled to monitor the dissection process (Figure 1C). As seen in the figure, the dissection was very precise, making it feasible to isolate subdomains within a given band, i.e., corresponding to individual genes or separating maternal from paternal chromosomal constituents.

A loss of resolution served as an indicator of possible chromatin absorption to the scanning probe. This can be seen by comparing the ESM images in Figure 1B and Figure 1C. A portion of the chromosome is also seen on the substrate just above the dissected region. The silicon nitride tips oxidize into silicon oxide during the manufacture process, creating a glassy surface that many biomolecules spontaneously adhere to. It is likely that DNA from many different sources could potentially contribute to the nucleic acid pool on the tip, resulting in a serious contamination problem. This is especially important with regard to the subsequent PCR amplification step in the protocol under development. However, in this test system, the overwhelming majority of DNA should come from the dissected region of the polytene chromosome which has 500 to 1000 parallel strands of DNA in register.

Preliminary experiments using PCR to amplify dissected material adhering to AFM tips with non-specific primers has indicated the presence of DNA. In contrast, both new tips and those used to lightly scan, but not

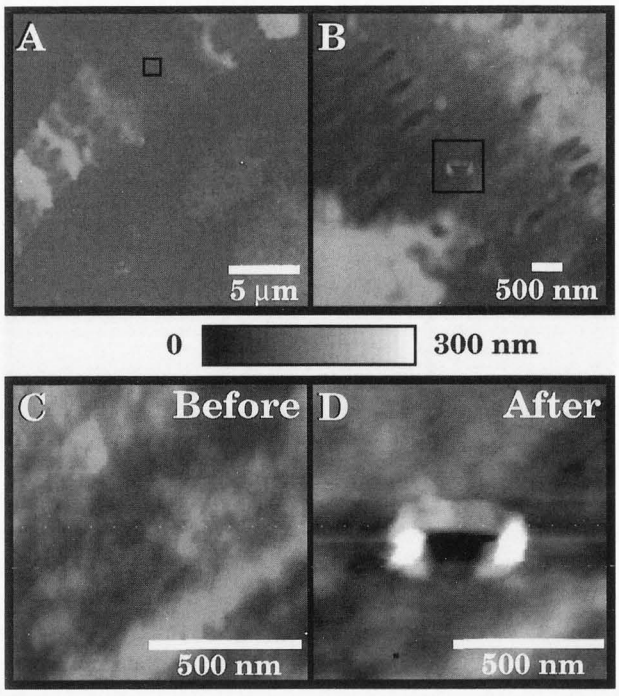

Figure 3. Flat, shallow regions of polytene chromosomes, such as interband regions, allow for nanometer length dissections that are limited only by the apical structure of the scanning probe. In this example from the 23C interband region of a *Drosophila* chromosome 2L arm, progressively higher magnifications are used to show the potential for precision cutting (**3A-3D**). Figures **3A** and **3C** are images prior to cutting and Figures **3B** and **3D** are after the cut. The cut here is 250 nm long and 50 nm wide, with chromosomal buildup appearing on both sides of the cut.

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dissect, the chromosome exhibited levels of amplified DNA above background (data not shown). Future efforts will be directed toward maintaining ultra-clean conditions including use of tip sterilization [18] and other established protocols [10] to minimize contamination problems and allow for dissection of single copy (non-polytene) genes.

Figure 2 is a detailed multi-sectional analysis of the height mode image in Figure 1C. Since the 1E band had swollen to approximately 400 nm in height, the shape of the tip can be seen in the geometric profile of the dissected region. The importance of the observation is that the dimension of the dissection was limited only by probe geometry. The pyramidal shaped probes can give exceptionally sharp cuts when the height of the specimen is small compared to the height of a typical probe (about 2 μm tall). Indeed, the base of the cut in Figure 2 in the narrow area is only 20 nm wide, about the limit of the width of dissection seen elsewhere [3, 4, 19]. Therefore, in regions where the height of the chromatin is small, such as interbands, exceptionally fine cuts can be made (Figure 3).

Figure 3A is a low magnification image of the 23C interband region from a 2L arm of a *Drosophila* polytene chromosome. The inset box represents the zoomed region seen in Figure 3B, where a small linear dissection is evident. The boxed region in this figure is further magnified in Figures 3C, prior to dissection, and Figure 3D, after dissection. The nano-dissection is 250 nm long by 50 nm wide. Notice that the chromosomal material appears to have built up on both sides of the fast scan termination points, common to AFM imaging in the contact mode. Evident in this Figure, and even more obvious in Figure 1, is swelling of the chromosomes.

We observed that during the microdissection process chromosomes swell and contract repeatedly as a function of hydration (Fig. 1). Between 20% and 70% relative humidity, the relative sample height increased 6 $\pm$ 3% (18 measurements of average band or interband height along an axis perpendicular to the chromosome length from 5 different chromosomes). We believe this effect is not due to the capillary layer alone because the relative increase of 8.2 nm is larger than experimentally observed capillary layer values of about 1 to 2 nm [18]. A striking difference in chromosome swelling was observed when submerged in physiological buffer. Under these conditions, the relative height increase was 450 $\pm$ 60% (17 measurements of average band or interband height along an axis perpendicular to the chromosome length from 5 different chromosomes). This swelling has been seen with human metaphase chromosome [2] and we tentatively attribute it to hydration of the nucleoprotein complex. Interestingly, after hydration for 24 hours, the average height decreased somewhat, which we suspect may be due to degradation of the sample. These data suggest that the higher order chromatin structure is maintained to some degree in dehydrated chromosomes. It may be possible to extract information about the nature of this complex folding problem by drawing correlative relationships between chromosome height and hydration, ionic, or other environmental states.

Conclusions

One of the powerful prospects of AFM is its utility as a nano-manipulation tool. We are currently developing protocols for the microdissection of chromosomal bands. We have been able to remove segments from band and interband regions of *Drosophila melanogaster* polytene chromosomes, at greater resolution than can be

achieved by conventional techniques. Preliminary efforts at PCR amplification of the dissected material suggest that we are able to amplify chromosomal DNA adsorbed onto a scanning probe apex. One near term objective of these studies is to generate hybridization probes by nano-dissection that can be used for high resolution mapping of genetic loci.

Preliminary studies show that chromosomes are structurally responsive to environmental conditions, such as hydration in phosphate buffered saline. These responses are repeatable, which implies that they are the consequence of a preserved higher order structure in the chromosome. Similar chromosomal studies have been conducted by Puppels *et al.* [15], employing combined AFM/Raman microspectroscopy. These studies explored the DNA and protein content of band and interband regions of polytene chromosomes and provide AFM images of comparable resolution. Our AFM studies of chromosomes have provided detailed ultrastructural information resulting from environmental influences and not detectable by light microscopy techniques.

Future work in this area may prove fruitful in unfolding the long standing question of how the genetic material is packaged three-dimensionally in chromosomes. The studies presented here begin to blend biomolecular and biophysical methods. As these intangible, but traditional, boundaries blur, powerful new approaches to genetic investigations will emerge.

Acknowledgments

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References

[1] Binnig G, Quate CF, Gerber C (1986) Atomic force microscope. Phys. Rev. Lett. **56**: 930-933.

[2] DeGrooth B, Putman C (1992) High-resolution imaging of chromosome-related structures by atomic force microscopy. J. Microsc. **168**: 239-247.

[3] Hansma H, Vesenka J, Siegerist C, Kelderman G, Morret H, Sinsheimer RL, Elings V, Bustamante C, Hansma PK (1992) Reproducible imaging and dissection of plasmid DNA under liquid with the atomic force microscope. Science **256**: 1180-1184.

[4] Henderson E (1992) Imaging and nanodissection of individual supercoiled plasmids by atomic force microscopy. Nuc. Acids Res. **20**: 445-447.

[5] Henderson E (1994a) Atomic force microscopy in basic and applied nucleic acid research. Clin. Chem. **40**: 653-653.

[6] Henderson E (1994b) Imaging of living cells by atomic force microscopy. Prog. Surf. Sci. **46**: 39-60.

[7] Henderson E, Haydon PG, Sakaguchi DS (1992) Actin filament dynamics in living glial cells imaged by atomic force microscopy. Science **257**: 1944-1946.

[8] Hoh JH, Hansma PK (1992) Atomic force microscopy for high resolution imaging in cell biology. Trends Cell Biol. **2**: 208-213.

[9] Hoh JH, Lal R, John SA, Revel J-P, Arnsdorf MF (1991) Atomic force microscopy and dissection of gap junctions. Science **253**: 1405-1408.

[10] Johnson DH (1990) Molecular cloning of DNA from specific chromosomal regions by microdissection and sequence-independent amplification of DNA. Genomics **6**: 243-51.

[11] Lal R, John SA (1994) Biological applications of atomic force microscopy. Am. J. Physiol. in press.

[12] Pardue ML, Gall JG (1975) Nucleic acid hybridization to the DNA of cytological preparations. Methods Cell Biol **10**: 1-16.

[13] Parpura V, Haydon PG, Henderson E (1993a) Three-dimensional imaging of living neurons and glia with the atomic force microscope. J. Cell. Sci. **104**: 427-432.

[14] Parpura V, Haydon PG, Sakaguchi DS, Henderson E (1993b) Atomic force microscopy and manipulation of living glial cells. J. Vac. Sci. Technol. A **11**: 773-775.

[15] Puppels G, Putman C, de Grooth B, Greve J (1992) Raman microspectroscopy and Atomic Force microscopy of chromosomal banding patterns. Proc. SPIE **1922**: 145-155.

[16] Putman CAJ, van der Werk K, de Grooth BG, van Hulst NF, Greve J, Hansma PK (1992) A new imaging mode in Atomic Force Microscopy based on the error signal. Proc. SPIE **1639**: 198-204

[17] Radmacher M, Cleveland JP, Frtiz M, Hansma H, Hansma P (1994) Mapping interaction forces with the atomic force microscope. Biophys. J. **66**: 2159-2165.

[18] Thundat T, Zheng X-Y, Chen GY, Sharp SL, Warmack RJ, Lindsay SM (1993) Characterization of atomic force microscope tips by adhesion force measurements. Appl. Phys. Lett. **63**: 2150-2152.

[19] Vesenka J, Guthold M, Tang CL, Keller D, Delaine C, Bustamante C (1993) A substrate preparation for reliable imaging of DNA molecules with a scanning force microscope. Ultramicroscopy **42-44**: 1243-1249.

[20] Wesley C.S, Ben M, Kreitman M, Hagag N, Eanes WF (1990) Cloning regions of the Drosophila genome by microdissection of polytene chromosome DNA and PCR with nonspecific primer. Nuc. Acids Res. **18**: 599-603.

Discussion with Reviewers

T.M. Seed: What are the major limiting factors of AFM dissection of biologic materials? What are the size limitations?

Authors: The major limiting factors of AFM dissection of biologic materials is primarily the tip shape. The precision is limited by a tip's aspect ratio and the height of the sample. Also influencing dissection is the force interaction between the substrate and the sample versus the lateral interaction force between the tip and the sample. Currently the greatest challenge is sample retrieval and steps are being taken in our lab to alleviate this problem.

H. Hansma: It would seem that the dissected DNA must be quite damaged, if dissections require several minutes of scanning at high force. How will this affect the applications envisioned for AFM dissection of chromosomes?

E. de Harven: The triumph would be, of course, to demonstrate practically the same sequence in several distinct experiments ... Until this is achieved (and it would be a fantastic achievement!), the devil's advocate could say that, at this point, the authors have demonstrated a very sophisticated method ... to damage chromosomes!

T.M. Seed: The PCR related work was mentioned but not described in any meaningful way. Without the PCR data being included, the reader is expected to accept the effectiveness of AFM microdissection of selected chromosomal regions as a matter of blind faith.

Authors: Because polytene chromosomes are naturally occurring multiple genes of DNA, the structural integrity of the chromosome is maintained by three-dimensional interaction with other DNA strands, unlike single stranded DNA. Thus, a higher scanning force is required to break the multiple layers of DNA. It is impossible to state at this time the amount of fragmentation or damming of the DNA. In principal, PCR amplification followed by gel electrophoresis should enable us to determine what size of DNA fragments have been recovered.

H. Hansma: In Figure 1C, the authors point out that material (DNA?) has been transferred to the substrate adjacent to the cut. Also, in Figure 3, it looks like much of the DNA is not transferred to the tip but is just pushed to the sides of the scan area. These observations seem to weaken the theory that dissected DNA will adhere to the AFM tip, which can be subsequently subjected to PCR.

Authors: We have made many cuts with different AFM tips. One of our criteria for detecting physioabsorbtion is the reduction in image resolution, due to contamination of the tip with sample. In the examples included in this paper, we selected images having very clean cuts and only marginal reduction in resolution suggesting less than complete adherence of the sample to tip. We have several examples where the resolution is reduced dramatically with no dissected sample to the side. Also, volumetric measurement of the remaining material shows it to be significantly less than the origiinal amount.

N.F. van Hulst: AFM images were taken in contact mode in dry air. Would tapping mode in liquid not be far more preferable?

Authors: Tapping mode in this instance is not preferable because higher forces are required to facilitate dissection. However, tapping mode could be useful in band identification prior to band dissection, because it would reduce the potential for tip contamination. This possibility is under investigation.

N.F. van Hulst: What does the dissection add to the observation and is it essential to absorb chromatin to the probe?

Authors: Our interests at this point are primarily in the recovery of DNA for PCR amplification. Absorption of material to the probe is currently the most promising method for the isolation of dissected material and is of vital importance in developing this technique further.

R.J. Warmack: The characteristics of an ideal tip for extracting DNA fragments would include a non-stick surface for imaging and a sticky surface during extraction. Is there a way around this dilemma for the Bioscope? Could a properly prepared (adherent) tip be lowered once onto a region of the chromosome that had been previously located by optical examination?

Authors: At present, we are examining the use of electron beam deposited (EBD) tips as potential low-stick surface for imaging while using standard silicon or silicon nitride (which oxidize to form a glass surface) as a sticky surface for DNA extraction. Unfortunately, EBD tips tend to break off rather easily when imaging tall structures such as polytene chromosomes.

R.J. Warmack: The large increases observed following hydration would indicate dramatic changes in conformation. Were the overall lengths and widths of the chromosome arms unchanged? How did ionic strength affect the dimensions?

Authors: Following hydration, increases in band widths (perpendicular to chromosome length) were observable but were complicated by tip contribution to the AFM images. Rough calculations suggest these later increases in dimensions are not of significance. Chromosomal band length were apparent but crowding of some

adjacent bands made quantification difficult. Significant overall chromosome length changes were not observed however. The effect of ion concentration on chromosomal structure was not measured but would seem likely to influence the higher order structure of chromosomes. No attempt was made to measure the ionic strength effects.

R.J. Warmack: How was the apparent height under physiological solution affected by changes in the applied cantilever force? Do you suspect significant deformation by the tip in either the dry or hydrated sample?

Authors: The effect of tip deformation on the chromosomes was not directly tested but steps were taken to maintain consistently low force throughout the imaging portion of the experiments and from sample to sample to minimize specimen damage. The samples are very robust in air and are not affected by the scanning forces we employed. In fluid there exists collateral evidence that the chromosomes will follow the motion of the tip and thus deform laterally to some extent. Still, the overwhelming height increase suggests a significant change in hydration state.